

WHAT IS CLAIMED IS:

Silvestri
1. A yeast which ferments xylose to ethanol,
comprising:

5 which ^{is} a yeast having genes integrated at each of multiple reiterated ribosomal DNA sites of the yeast, said genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase.

10 2. The yeast of claim 1 which also ferments glucose to ethanol.

3. The yeast of claim 2 which is *Saccharomyces*.

15 4. The yeast of claim 3 wherein said sites are non-transcribed DNA sites.

20 5. The yeast of claim 1 wherein the genes are fused to non-glucose-inhibited promoters and the yeast simultaneously ferments glucose and xylose to ethanol.

6. The yeast of claim 5 wherein the promoters do not require xylose for induction.

25 7. The yeast of claim 3 wherein the genes are fused to non-glucose-inhibited promoters and the yeast simultaneously ferments glucose and xylose to ethanol.

30 8. The yeast of claim 4 wherein the genes are fused to non-glucose-inhibited promoters and the yeast simultaneously ferments glucose and xylose to ethanol, the promoters also not requiring xylose for induction.

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9. The yeast of claim 6 wherein the xylose reductase and xylitol dehydrogenase genes are from natural yeast which ferment xylose to ethanol.

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10. The yeast of claim 9 wherein the natural yeast are *Candida Shehatae*, *Pichia stipitis* or *Pachysolen tannophilus*.

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11. The yeast of claim 9 wherein the xyulokinase gene is from a yeast or bacteria.

12. The yeast of claim 11 wherein the xyulokinase gene is from *Candida Shehatae*, *Pichia stipitis*,
15 *Pachysolen tannophilus*, *Saccharomyces cerevisiae*,
Schizosaccharomyces pombe, or *Escherichia coli*.

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13. The yeast of claim 1 having said genes integrated ~~at~~ at least about 10 ribosomal DNA sites of the yeast.

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14. A method for integrating multiple copies of exogenous DNA into reiterated chromosomal DNA of cells, comprising:

(a) transforming the cells with a replicative and integrative plasmid having exogenous DNA including a first selection marker; and

(b) repeatedly replicating the cells from step (a) to produce a number of generations of progeny cells while
30 selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of the

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progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA.

15. The method of claim 14, wherein the plasmid DNA
5 also includes a second selection marker for selecting cells which include the plasmid.

10 16. The method of claim 14 wherein the cells are yeast or eukaryotic cells, and wherein the method further includes the step of repeatedly replicating the progeny cells from step (b) to produce a number of generations of progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells
15 and recover yeast cells each containing multiple copies of the exogenous DNA integrated into its chromosomal DNA.

20 17. The method of claim 16 wherein the cells are yeast cells and the exogenous DNA includes genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, which also serve as the first selection marker.

25 18. The method of claim 14, which comprises:
(i) transforming yeast cells with a replicative plasmid having exogenous DNA including a selection marker, the exogenous DNA being flanked on each end by a DNA sequence homologous to a reiterated sequence of DNA of the host;

30 (ii) repeatedly replicating the transformed yeast cells from step (i) to produce a number of generations of progeny cells while selecting for cells which include the

selection marker, so as to promote the retention of the replicative plasmid in subsequent generations of the progeny cells and result in progeny cells each containing multiple integrated copies of the exogenous DNA; and

- 5 (iii) replicating the progeny cells from step (ii) to produce a number of generations of progeny cells in the absence of selection for cells which include the selection marker, so as to promote the loss of the plasmid in subsequent generations of progeny cells and
- 10 recover yeast cells each containing multiple copies of the exogenous DNA integrated into its chromosomal DNA.

19. Yeast cells produced by the method of claim 18.

- 15 20. The yeast cells of claim 19, wherein the exogenous DNA includes genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, and the yeast cells ferment xylose to ethanol.

- 20 21. The yeast cells of claim 20, wherein said genes are fused to non-glucose-inhibited promoters which do not require xylose for induction, and wherein the yeast cells ferment glucose and xylose simultaneously to ethanol.

- 25 22. Yeast cells according to claim 21 which substantially maintain their capacity to ferment xylose to ethanol when cultured under non-selective conditions for at least 20 generations.

- 30 23. A yeast which ferments xylose to ethanol, comprising:

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5 a yeast having multiple copies of exogenous DNA integrated into chromosomal DNA of the yeast, the exogenous DNA including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase fused to non-glucose-inhibited promoters, the yeast fermenting glucose and xylose simultaneously to ethanol and substantially retaining its capacity for fermenting xylose to ethanol for at least 20 generations when cultured under non-selective conditions.

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24. The yeast of claim 23, wherein said promoters do not require xylose for induction

15 25. A yeast which ferments xylose to ethanol, comprising:

20 a yeast having multiple copies of an introduced DNA containing genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase, the yeast fermenting xylose to ethanol and substantially retaining its capacity for fermenting xylose to ethanol when cultured under non-selective conditions for at least 20 generations.

25 26. The yeast of claim 25, wherein the promoters do not require xylose for induction

30 27. A method for fermenting xylose to ethanol, comprising fermenting a xylose-containing medium with a yeast of claim 1, 22, 23, 24, 25 or 26, to produce ethanol.

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28. A plasmid vector for integrating an exogenous DNA sequence including a first selection marker into chromosomal DNA of a target yeast cell, the plasmid vector containing a functional yeast DNA replication origin and the exogenous DNA flanked on each end by a DNA flanking sequence which is homologous to a reiterated ribosomal DNA sequence of the target yeast cell, the plasmid further including a second selection marker in a position other than between the DNA flanking sequences.

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29. A plasmid vector for integrating an exogenous DNA sequence into a yeast to form stable integrants which ferment xylose to ethanol, the plasmid vector containing a functional yeast DNA replication origin and exogenous DNA including genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase flanked on each end by a DNA flanking sequence which is homologous to a reiterated DNA sequence of the target yeast cell.

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30. A method for forming cells having multiple integrated copies of an exogenous DNA fragment, comprising:

replicating cells having reiterated genomic DNA and which contain a replicative and integrative plasmid containing the exogenous DNA to produce multiple generations of progeny cells while selecting for cells which include the selection marker, so as to promote the retention of the replicative and integrative plasmid in subsequent generations of the progeny cells and produce progeny cells having multiple integrated copies of the exogenous DNA.

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